

NITROGEN EXCRETION IN TWO
PULMONATE LAND SNAILS

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INTRODUCTION

Of the major classes of biological materials, sugars, fats, and proteins, only proteins cannot be stored. Ingested proteins, or protein from breakdown and turnover of body cell components, must be degraded and the products used or discarded. Products of this degradation are amino acids, which are transaminated or deaminated. If an animal is a carnivore, very large amounts of protein nitrogen are ingested, and much of this nitrogen must be eliminated. Another source of waste nitrogen is catabolism of nucleic acids; the major degradation products of these are purines and pyrimidines.

Several nitrogen-containing compounds have been found commonly in animal tissue, body fluids, and excretions. Free amino acids are found in invertebrates (Awapara, 1962), with fresh-water and land invertebrates having a lower concentration of amino acids than marine invertebrates (Potts, 1967). Molluscs have been reported to excrete large amounts of amino acids. Lymnaea stagnalis excretes 28% of its nitrogen as amino acids (Spitzer, 1937), and Helix pomatia, 5 - 8% (Delaunay, 1931).

Ammonia is produced by transamination and deamination of amino acids. Because of its toxicity, it must be excreted

at once, or converted to another, less toxic, substance. Some aquatic molluscs are known to excrete large amounts of ammonia. Delaunay (1925) found that ammonia represents 65% of the non-protein nitrogen of the urine of Sepia officinalis. Land pulmonates have been reported to have larger amounts of ammonia in their blood than do fresh-water pulmonates (Delaunay, 1931), but excrete less ammonia (Spitzer, 1937).

Urea is found in large amounts in urine of vertebrates. Man excretes 85 - 90% of his waste nitrogen as urea (Folin, 1905). Invertebrates produce much smaller amounts of urea. Arginine is a major amino acid in plants, forming about 14% of the total amino acids (Baldwin and Needham, 1934). Since the action of arginase on arginine results in urea formation, and land molluscs are known to contain a great deal of arginase activity (Baldwin, 1935; Gaston and Campbell, 1966), presumably they could excrete urea as a result of dietary arginine. Early authors, notably Delaunay (1931), Needham (1935), and Baldwin (1947), believed urea was an important excretory product of land snails, but more recently it has become apparent that urea represents only a very small percentage of waste nitrogen (Jezewska, Gorzkowski and Holler, 1963a; Campbell and Speeg, 1968).

Purines, especially uric acid, have been found in a wide variety of molluscs. Strohl (1914) suggested that

uric acid is typically an excretory product of adult gastropods. Xanthine and guanine have also been found in the kidney and excreta of gastropods (Jezewska, et al., 1963a; Lee and Campbell, 1965).

The usual method for disposing of waste nitrogen is elimination in the urine, but gastropods have the additional capacity to store materials in their kidneys. Jacobson (1820) showed that the kidney of H. pomatia contains largely uric acid, and Marchal (1889) purified and measured this uric acid. He reported that H. pomatia kidney contains an average of 7 mg of uric acid. Delaunay attempted measurements of nitrogenous materials from both kidney and urine of H. pomatia. In an often-quoted study, Delaunay (1931) reported that urea made up 20% of the excretory products. He also found amino acids and small amounts of uric acid in water extracts of excreta and much uric acid in the kidney.

However, Delaunay recognized that his collection procedures did not recover all the uric acid. His water extract of kidneys left about 80% of the purines un-extracted (Jezewska, et al., 1963a). He collected "liquid excreta" by keeping the snails partially immersed in water, and analysing the water for nitrogenous materials, again leaving most of the purines behind.

The uricotelic nature of H. pomatia was determined by Baldwin and Needham in 1934. Identification of molluscan excretory products was largely neglected until 1963, when

Jazawska, et al., (1963a) analysed H. pomatia kidneys and excreta. They found that purines accounted for 91 - 98% of the nitrogen in the kidney and 96 - 102% of the nitrogen in voided excreta. Three purines were found: uric acid, guanine, and xanthine, the latter two often being in excess of uric acid. The same three purines were found in Otala lactea (Lee and Campbell, 1965).

The pattern of nitrogenous excretory products of mollusca has been reported to be determined by several factors. The most widely known theory is that of Needham (1935) which holds that the major nitrogenous excretory product of the adult can be correlated with the environment of the early embryo. Aquatic embryos, having access to unlimited water, are able to excrete toxic ammonia. Land snails, however, spend their embryonic life in cleidoic eggs. The adults should excrete mainly uric acid, which is easily stored or excreted in a solid form, conserving water. This theory, holding well for vertebrates, also seems to be valid for molluscs. Sloan (1956), however, studied two closely related fresh-water snails, Ceratodes cornuarietis and Pomacea paludosa. C. cornuarietis embryos develop in fresh-water; P. paludosa eggs are cleidoic, deposited above the water level. The cleidoic egg of P. paludosa accumulates uric acid, the egg of C. cornuarietis excretes ammonia. Adults of both species excrete mainly ammonia. Thus it is apparent that the excretory product of the embryo

is not necessarily that of the adult. Duerr (1967) also questions Needham's theory, suggesting instead that uricotelism is a phylogenetic trait among the gastropods, and is not affected by environment.

Jezewski, et al., (1963b) have found that the pattern of purine metabolism is affected by activity. During estivation, large amounts of uric acid accumulate in the kidney of H. pomatia. Active snails store and void proportionately more xanthine and guanine. Type and amount of food ingested may possibly affect the pattern of nitrogen waste materials. Dresel and Moyle (1950) found that land isopods and amphipods excrete a large percent of their nitrogen waste as ammonia, and some fresh-water forms have large amounts of uric acid in their tissues. They suggested some sort of "suppression" of nitrogen metabolism in land forms. Recently Sloan (personal communication) confirmed ammonotelism in amphipods, and indicated that gaseous ammonia is a metabolic end-product of amino acid metabolism. The quantity of ammonia excreted seems a function of diet, but there is no indication of suppression of nitrogen metabolism. O. lactea is reported to excrete gaseous ammonia, and the amount is apparently greater in estivating snails than active ones (Speeg and Campbell, 1968).

Few attempts have been made to draw up a complete balance sheet of nitrogenous waste products of a molluscan species. Delaunay was successful in a study of S. officinalis

(1925), but not as successful with H. pomatia (1931).

Albritton (1954) gives tables containing data gathered from various sources. The species of land snails for which data are given are A. ov. aspirodon, L. ax. per. tis, and L. pomtia. The data must be carefully interpreted, however, since they were obtained by several different methods, using different techniques and were obtained from a variety of conditions, creating inconsistencies. Baldwin (1955) was able to account for 50% of the nitrogen excretion of H. pomatia as:

Ammonia 13.7%

Urea 20.0%

Uric acid 10.0%

Amino acids, creatinine, and other compounds 6%.

Jozewski, et al. (1963a) found that in L. pomatia, over 90% of total nitrogen in the kidney and voided excreta consists of uric acid, xanthine, and guanine. Ammonia, urea, and amino acids were not detected in kidney or excreta.

Due to the paucity of this type of information on land snails, it was felt that there was a need for a systematic identification and measurement of the major nitrogenous components of land snail excreta and kidney, with the goal of determining a balance sheet of the organism. It is necessary to have a quantitative picture of the excretion of nitrogenous waste of the snail before attempting to develop, compare, and select their excretions.

The species chosen for this analysis were Euglandina
rossa (Perrussat) and Metamphix variabilis Baker. Euglandina
 is a large carnivorous pulmonate. Metamphix, also a
 pulmonate, is a small scavenger and is the natural prey
 of Euglandina. These two species give an opportunity to
 compare the nitrogenous excretory products of a predator
 and its prey, and also those of a carnivore and a scavenger.

MATERIAL.

Most of the specimens of H. grandis used in this study were collected in a hillside hardwood hammock on the University of Florida campus in Gainesville, Florida. The main trees in the area are oaks, with a few magnolias. The snails were found under decaying logs and leaves on the ground. Other specimens were collected near or on the sides of houses in the city of Gainesville, itself. In a few instances, clutches of eggs were found and hatched in the laboratory, and the snails raised to usable size. The Hesperomys were collected exclusively from the swampy hammocks, under leaves or partially buried in the humus.

The Richardsoni were kept in individual glass finger-bowls on drop filter paper. They were fed 3-4 times a week. Food consisted of terrestrial and aquatic paludonates, with aquatic paludonates predominating since they were easier to obtain in quantity.

Hesperomys were used as soon after collection as possible since they did not feed or grow well in the laboratory. When held for a few days, they were kept in large fingerbowls on drop filter paper with about 20 snails to a bowl. Both species were kept in a well-aerated environment at 23°C and 80% relative humidity. The snails were

active under these conditions, usually extended partway out of their shells when not actually moving about.

Englandina excreta emerge from a slit just anterior to and continuous with the pneumostome as a cylindrical ribbon of white pasty material, physically separate and easily distinguishable from the jet black fecal material. The excreta dry rapidly when exposed to air. Only fresh moist excreta were collected for this study in order to minimize bacterial degradation of waste products. Excreta were either used fresh, or frozen at -20 C for future analysis.

Mesomphix were held for 2 - 3 days after collection in small finger bowls to collect all waste products, which were removed periodically and frozen at -20 C for future analysis.

Kidneys of both species were carefully separated from surrounding tissues, removed, and frozen at -20 C.

METHODS

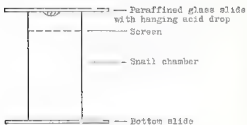
Ammonia

a) Gaseous Ammonia

Two methods were used to determine whether the two species studied release any gaseous ammonia into the atmosphere. In the first method, a modified Conway diffusion technique (Conway, 1933), a snail was placed in a small glass cylinder (Figure 1). Near one end was cemented a piece of plastic screen to keep the snail restricted from that end of the cylinder. The open end of the cylinder was closed with a glass slide, sealed onto the cylinder with a paraffin-cerclene mixture. The screened end was closed with a paraffin-coated slide on which a hanging drop of HCl with known normality was placed. Any ammonia given off by the snail would be collected in the HCl which could be titrated and the ammonia determined. Snails were left in the cell for 2 hours. Recovery of ammonia standards averaged 96.8%.

The second method was adopted from Speng and Graybe (1963). Air was bubbled through three flasks containing H_2^{18}O to saturate any ammonia in the incoming air. The air then passed through a small glass cylinder containing a small amount of HCl solution. The ammonia released from the snail would be collected in the HCl solution.

Figure 1. Diffusion chamber for detection and measurement of gaseous ammonia.



bubbler into 0.05 N HCl. After 12 hours, the HCl was titrated to determine the ammonia. Recovery of known amounts of ammonia placed in the small chamber was 100%.

b) Dissolved Ammonia

Excretory and kidney ammonia content was determined by the method of Shaw and Beadle (1949) and, specifically, as modified by Gregg (1950). Half of the material to be analysed was dried to determine the water content. The remainder was analysed while wet to avoid driving off ammonia in the drying process.

The method of analysis involved the use of a small (0.5 ml) glass chamber, which was to be sealed with a paraffin-coated glass slide. The slide was prepared by cutting microscope slides in halves, and coating them with melted paraffin. When cool, the paraffin provided a hydrophobic surface to which could be applied a 6.7 μ l drop of 0.05 N HCl to absorb ammonia during the distillation process to follow. The slide was sealed to the glass chamber during distillation by a grease made by mixing melted paraffin and vaseline to a thick consistency.

The wet excreta were extracted with 0.01 N phosphate buffer, pH 6.8. A 10.6 μ l aliquot of the buffer extract was delivered into the glass diffusion chamber, and 20 μ l of 20% NaOH was added to distill off any ammonia present. Immediately upon addition of the NaOH, the paraffined glass slide with the drop of acid was inverted and sealed onto the

top of the chamber. After 3 hours the drop of acid was titrated to determine the ammonia content. Recovery of known amounts of NH_3 was averaged 99.0%.

Urea

Urea was analyzed by first degrading it to ammonia with urease (glycerol extract [Koch], from W. H. Curtin and Co.), then measuring ammonia as before. Recovery was 90.0%.

Purines

Material to be analyzed for purines was homogenized in 0.01 N NaOH in a ground-glass homogenizer. Samples of the homogenate were placed on glass thin-layer chromatographic plates spread with Alumina Grade G obtained from Research Specialties Company, Richmond, Calif. The plates were developed in isopropylalcohol (10:3). Spots were visualized using a 100 watt short wave ultra violet lamp producing a wavelength of 254 m μ . The spots were identified tentatively by H_2 , and were then eluted with four different solvents: a) 0.01 N Borate buffer, pH 8.3, b) 0.01 N NaOH, c) 0.1 N HCl, and d) 0.1 N phosphoric acid, pH 6.8. Ultraviolet spectra of the eluates were compared with those of known purines. Ultra violet spectra were obtained with a Bausch and Lomb Model 20 Recording Spectrophotometer. After spots were identified, purines were eluted, recovered and for comparison of the procedure for measurement.

Aliquots of homogenates were placed on Whatman No. 1 filter paper and developed in isopropanol-H₂O (10:3). After development, the spots were located by viewing under ultra-violet light as before and were eluted with distilled water. The optical densities at the spectral maxima were obtained and compared with standard curves of known purities. Recovery of 80 - 85% was routinely obtained. Results were adjusted to correct for recovery.

Amino Acids

a) Identification

Amino acids were identified according to the method of Shiralipour, Harris, and West (1969).

Material to be analyzed for amino acids was homogenized in a ground glass homogenizer with 95% ethanol. Samples of the ethanol extract were spotted on Whatman Chromagram Prespread Chromatography Sheets (Sheet 606 X, Cellulose with 1% Phosphoric Acid Indicator). Components of the extract were separated two dimensionally. Solvents used were:

first dimension - isopropanol:formic acid:
distilled water (80:4:20)

second dimension - tert.-butanol:methyl-
ethyl ketone:10% aq. distilled acid (10:10:10).

The chromatograms were allowed to develop in the first solvent for 16 hr., then removed and allowed to dry for 24 hr.

room temp, heated at 65 C for 15 min, and cooled for 15 min. The chromatogram then was placed in the second solvent and allowed to develop. After 6 hr it was removed and dried in air overnight.

Before location of the spots, the chromatogram was heated at 65 C for 15 min and then cooled. The spots were located by spraying with 0.5% ninhydrin in acetone and heating for 10 min at 65 C.

b) Estimation of Total Amino Acids

Although the amino acids were separated for qualitative identification, no attempt was made to determine the amount of each individual amino acid present. Rather, samples were analysed for total amino acid content.

Material to be analysed was homogenized in a ground glass homogenizer with 95% ethanol. Aliquots of the ethanol extract were spotted on thin-layer chromatography plates, using Silica Gel G as the absorbent. Plates were developed in n-butanol-acetic acid:water (60:20:20). Individual amino acids were identified by comparison of R_f with that of known amino acids. Measurement was done by elution from the thin-layer chromatography plates with distilled water and reaction with ninhydrin (Moore and Stein, 1954). The reaction was carried out as follows: 2 g of ninhydrin and 0.3 g of hydrindantin were dissolved in 75 ml of methyl cellosolve without incorporating bubbles. Twenty-five ml of 4 N sodium acetate buffer (pH 5.5) were added and the

resulting reddish reagent solution was immediately transferred to a dark-glass reservoir bottle and stored in the refrigerator. The pH 5.5 buffer was made by adding 200 ml of water to 272 g of sodium acetate-3 H₂O and stirring the mixture on a hot water bath. The solution was cooled to room temp, 50 ml glacial acetic acid added, and the volume made up to 500 ml. It was stored at 4 C.

One ml of sample containing amino acids to be measured was added to 1 ml of the reagent, the capped tube shaken (<10 sec.) and heated for 15 min in a boiling water bath. Five ml of diluent (50% ethanol) were added to each tube, cooled to 30 C, and the tubes were shaken in a rack for <30 sec. Absorbance was read at 570 mμ against a blank of water. The unknowns were compared with a standard curve made with leucine (0.05 - 0.2 mM), and results reported as "leucine equivalents". Recovery of known amounts of amino acids averaged 90.0%.

Total Non-Protein Nitrogen (TNPN)

It was found that the usual methods for deproteinization were not applicable for this study, since most call for addition of acid, such as trichloroacetic acid. Upon acidification of a solution, any purines present were precipitated and lost along with the protein. Therefore it was decided to separate protein from the non-protein components, measure the protein nitrogen, and subtract the protein

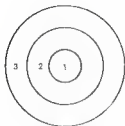
nitrogen from the total nitrogen to give TNM. Separation of the protein from other components was accomplished by Sephadex column chromatography. Sephadex G-25 (medium), obtained from Pharmacia Fine Chemicals Inc., Piscataway, N. J., was used in a column of 12 mm diameter and 200 mm height. A test solution containing bovine serum albumin and uric acid was found to be separated completely by this column. Mucous from the snails studied gave a characteristic ultra-violet spectrum and could therefore be detected in the fractions collected from the column.

The material to be analysed was homogenized in 0.01 N NaOH and centrifuged at 12,000 RPM in a Servall Type SS-1 Superspeed Angle Centrifuge. A 0.05 ml aliquot of the supernate was placed on the Sephadex column for fractionation. Fractions containing only protein were collected and the nitrogen content was determined by a Kjeldahl method, as was the nitrogen content of aliquots of the unfractionated supernate.

The material to be analysed for nitrogen was placed in a 10 ml volumetric flask with a small glass bead to prevent bumping of the fluid in the intense heat used. One ml of 20% H_2SO_4 and a small amount of selenium metal (Koch and McMeekin, 1924) were added. The flask was placed in an angled hole in a large aluminum block on a hot plate. The fluid was heated to a temperature in excess of 200 C for 6 hours. It was then allowed to cool to room

temperature, 4 drops of 30% H_2O_2 were added to oxidize all remaining material, and the flask was heated again for 20 min. It was then cooled, and the contents diluted to 10 ml. The Conway diffusion method was used to determine the ammonia content of the flasks. One-ml aliquots from each flask were pipetted into the reaction chamber of a Coleman Microdiffusion dish (Figure 2), 1 ml 0.05 N HCl containing methyl red was added to the collector chamber, 1.5 ml 20% NaOH pipetted into the sealing chamber and 1.0 ml of 20% NaOH pipetted into the reaction chamber. The dish cover was quickly placed over the dish and the dish was allowed to stand for 12 hours. The dish cover was then removed and the HCl titrated with a Coleman Microtiter to determine the ammonia content. Recovery of known ammonia was 98.8%. Recovery of known uric acid averaged 83.6%. Results of determinations were adjusted for recovery. Protein nitrogen was subtracted from total nitrogen to give TPA.

Figure 2. Corrugated Diffusion Cell. A - top view
1) Collector Chamber 2) Reaction Chamber
3) Sealing Chamber B = side view



A



B

RESULTS

Results of analyses on kidney and excretory material are summarized in Table 1. No material identifiable as excretory in origin was found for Mesomphix vulgaris.

Ammonia

Dissolved ammonia was found in all material studied, also in very small amounts. Ammoniac nitrogen represents a larger percentage of the TNN in Eurylandina kidney than does urea ($p < 0.05$, t test), but in Eurylandina excreta as Mesomphix kidney there is no significant difference ($p < 0.20$). No gaseous ammonia was detected in either species by any method used. Recovery of known added ammonia was 100.0%.

Urea

Urea was present in all material studied, but in very small amounts. Results are given in Table 1.

Parines

Free parines excreta consist of white, etc., immediately aggregating in presence of parines. Kidneys of parines and parines are cream colored. Thin layer chromatography of the kidney extracts of parines kidney and excreta and parines kidney and excreta revealed only two spots in order of increasing R_f . The spots are labeled as follows:

TABLE 1

DEPOSIT WITHIN CONVEYER OF KIDNEY AND EXCRETA

Species	Interval	URIC ACID			GLYCINE								
		ug. N/g dry wt.			mg. N/g dry wt.								
		$\bar{x}$	SE	(N)	$\bar{x}$	SE	(N)	$\bar{x}$	SE	(N)			
C. elegans		18.7 ± 6.1 (9)			42.9 ± 3.6 (9)			18.5 ± 1.8 (9)			22.0 ± 2.2 (9)		
	range:	17.2-26.1			23.6-50.1			9.3-25.2			1.7-32.4		
C. elegans		75.1 ± 12.6 (8)			59.9 ± 9.6 (8)			10.8 ± 2.2 (8)			8.0 ± 1.2 (8)		
	range:	37.2-143.2			40.6-73.6			2.9-20.9			2.4-10.2		
C. elegans		223.7 ± 12.4 (7)			82.9 ± 4.6 (7)			18.7 ± 4.0 (7)			6.9 ± 1.5 (7)		
	range:	183.4-255.3			66.0-95.6			2.9-31.1			10.0-11.5		

TABLE 3
(cont)

Species	Material	URICA						AMMONIA						
		mg. %/g d.f. wt.			d. error			mg. %/g d.f. wt.			d. error			
		$\bar{x}$	SE	(n)	$\bar{x}$	SE	(n)	$\bar{x}$	SE	(n)	$\bar{x}$	SE	(n)	
P. n. n. 2														
		2.2 ± 0.8	(3)	2.5 ± 0.8	(8)	3.4 ± 0.9	(8)	3.8 ± 1.0	(8)					
	kidney range	0.6-6.4		0.8-7.1		1.9-9.1		1.2-9.2						
P. n. n. 3														
		1.4 ± 0.2	(3)	1.3 ± 0.3	(8)	3.6 ± 0.8	(8)	2.3 ± 0.4	(5)					
	kidney range	0.6-2.0		0.4-2.6		0.7-6.4		0.7-4.3						
P. n. n. 4														
		1.0 ± 0.2	(5)	1.1 ± 0.3	(6)	1.4 ± 0.7	(6)	0.5 ± 0.3	(6)					
	kidney range	0.7-5.5		0.3-2.0		0.8-4.8		0.0-1.8						

TABLE 1
(cont)

Treated Material	A-IND ACIDS (leucine equivalents)			TIPO		
	MEAN $\pm$ SD (N)	SE	(N)	MEAN $\pm$ SD (N)	SE	(N)
A-IND ACIDS						
	24.5 ± 5.4 (9)			24.7 ± 5.4 (9)		
	range 1.4-38.0			0.0-32.4		
TIPO						
	52.7 ± 12.7 (9)			52.7 ± 12.7 (9)		
	range 24.3-72.6			23.6-72.7		
A-IND ACIDS						
	10.3 ± 3.4 (10)			10.3 ± 3.4 (10)		
	range 2.3-24.6			7.5-24.7		
TIPO						
	57.8 ± 12.7 (9)			57.8 ± 12.7 (9)		
	range 24.3-72.6			23.6-72.7		
A-IND ACIDS						
	0.6 ± 0.1 (8)			0.6 ± 0.1 (8)		
	range 0.2-1.5			0.3-1.3		
TIPO						
	270.1 ± 5.5 (7)			270.1 ± 5.5 (7)		
	range 245.1-286.2			245.1-286.2		

chromatographed identically with guanine and uric acid, respectively. Ultra-violet spectra of eluates of the spots using four different solvents are shown in Figures 3 - 8, confirming the presence of guanine and uric acid. No other purines were detected in extracts of kidneys or excreta of either species studied. Uric acid represents the major portion of T₁: 43% in Mesosphix kidney, 6% in Euglandina kidney, and 83% in Euglandina excreta. Guanine represents a significant but much less plentiful component.

Amino Acids

a) Qualitative Results

Several amino acids were found in the kidneys and excreta (Figure 9). Fewer ninhydrin-positive spots were found on thin-layer chromatograms of Euglandina excreta than those of kidneys of the two species analysed. Amino acids present in Euglandina kidney were, in decreasing order of intensity of ninhydrin staining: tyrosine, histidine, arginine and lysine, homoserine, phenylalanine, norleucine, isoleucine, glutamine, serine, glycine, and valine. Mesosphix kidney contained, in decreasing order: tyrosine, phenylalanine, isoleucine, leucine, serine, glutamic acid, glycine, arginine and lysine, norleucine, histidine, and valine. Euglandina excreta contained tyrosine, histidine, lysine and arginine, isoleucine, leucine, glutamine, and serine.

Figure 3. Ultra violet spectra of known uric acid
and unknown compound of Group 15 kidney.
Solvents: A = 0.06 N NaOH; B = 0.06 M
borate buffer, pH 8.9; C = 0.1 M
phosphate buffer, pH 6.8; D = 0.1 N HCl.
———— Known uric acid;
- - - Unknown compound.

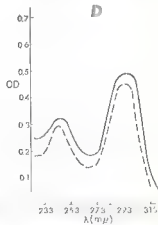
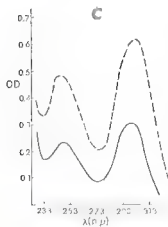
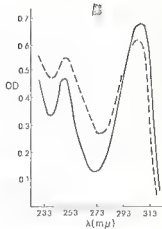
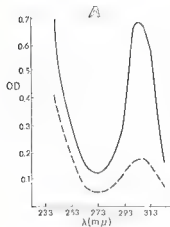


Figure 4. Ultra-violet spectra of known guanine and unknown compound of *Mesomphix* kidney.
 Solvent: A = 0.06 M borate buffer, pH 8.9; B = 0.06 M phosphate buffer, pH 6.8; C = 0.1 M phosphate buffer, pH 6.8; D = 0.1 N HCl.
 ————— Known guanine;
 - - - - - Unknown compound.

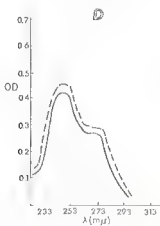
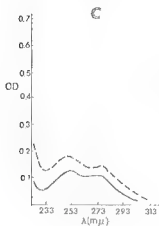
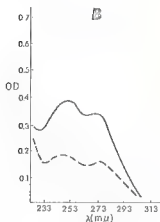
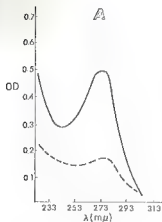


Figure 5. Ultra-violet spectra of a o-malleic acid and unknown compound of *Euglandina* kidney.
 Solvents: A = 1.0% o-malleic acid in 0.01 M borate buffer, pH 8.9; C = 0.1 M phosphate buffer, pH 6.8; F = 0.1 M HCl.
 ———— o-malleic acid.
 ---- Unknown compound.

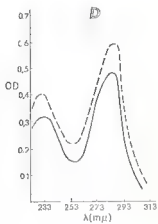
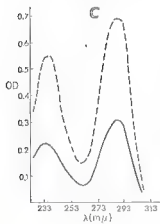
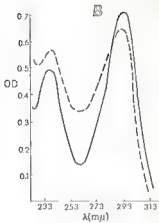
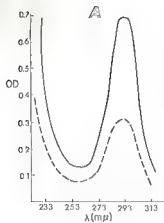


Figure 6. Ultra-violet spectra of known guanine and unknown compound of *Euglandina* kidney.
 Solvents: A = 0.06 N NaOH; B = 0.06 M borate buffer, pH 8.9; C = 0.1 M phosphate buffer, pH 6.8; D = 0.1 N HCl.
 ——— Known guanine;
 --- Unknown compound.

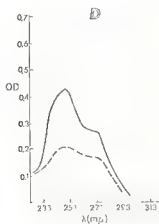
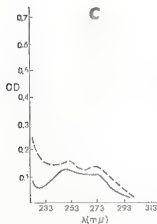
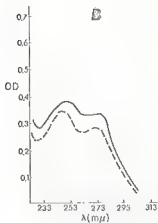
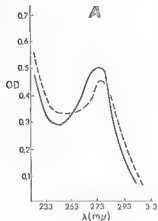


Figure 7. Ultra-violet spectra of known uric acid and unknown compound of *Euglandina* excreta.
 Solvents: A = 0.06 M NaOH ; B = 0.06 M borate buffer, pH 8.9; C = 0.1 M phosphate buffer, pH 6.8; D = 0.1 M H_2O .
 ————— Known uric acid;
 - - - - - Unknown compound.

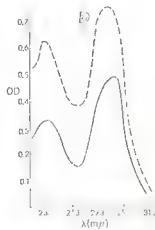
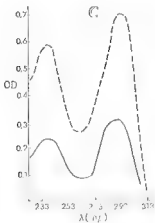
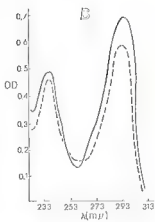
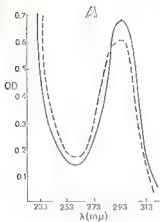


Figure 8. Ultraviolet spectra of an unknown compound of *Bufo* excreta.
 Solvent: A = 0.1 M borate buffer, pH 8.9; C = 0.1 M phosphate buffer, pH 6.8; D = 0.1 N HCl.
 — Known guanine;
 -- Unknown compound.

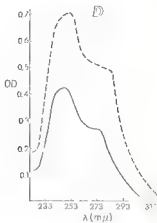
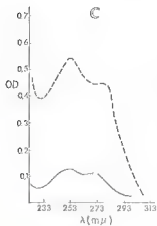
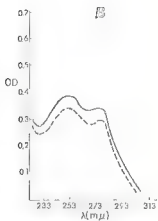
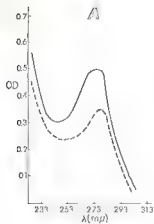
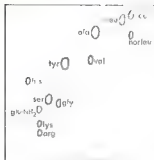
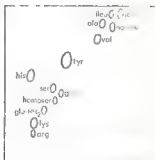


Figure 9. Two-dimensional thin-layer chromatograms showing patterns of amino acids.

A = Microphis kidney; I = Eschschoria
kidney, C = Paralichthys ocellus.



A



B



C

b) Quantitative Results

Amino acids of P. lunatus and Paralomis kidney, represented 1% and 2% of the TPN, respectively. Here then 1% of the TPN of Paralomis excreted as amino acid nitrogen (Table 1).

TPN

Paralomis kidney contained widely varying amounts of TPN, but averaged 50 mg/g dry wt. Paralomis kidney contained nearly twice as much TPN, 158 mg/g dry wt, and was somewhat less variable. Paralomis excreta contained 270 mg TPN/g dry wt. The range of this fraction is small, and indicates a fairly consistent composition of the excreta.

Protein in Excreted Excreta

The protein content of Paralomis excreta averaged 7.3 mg N/g dry excreta (SE = 1.2, N = 18).

Total Nitrogen Excretion of P. lunatus

An average of 1.6 mg of protein nitrogen found for each excretion of P. lunatus (N = 8 SE = 1.0, range = 1.6 - 10.2 mg N).

A summary of all quantitative results is given in Table 2.

TABLE 2

BALANCE SHEET OF NON-PROTEIN NITROGEN CONTENTS OF KIDNEY AND EXCRETA

Species	Material	Uric Acid % dry wt	Guanine % dry wt	Urea % dry wt	Ammonia % dry wt	Amino Acids % dry wt	Total % dry wt
<u>Paraperla</u> <u>flavipes</u>	kidney	42.9	22.0	2.5	1.2	24.7	93.3
<u>Paraperla</u> <u>flavipes</u>	kidney	55.9	8.0	1.1	2.3	19.3	86.6
	excreta	82.0	6.0	1.	0.5	0.5	92.0

DISCUSSION

It is interesting that Mesomphix produced no detectable excretory material. This finding, while not expected, may be quite common among species of small pulmonates. Most small snails appear to have a life span of only 1 year. Boycott (1934) found that all small British snails he studied were annual, with a life span of 9 - 15 months. Larger species, such as H. pomatia, had a life span of more than 3 years.

A terrestrial species with a short life span of 1 year might conserve water and energy by excreting nothing. A material such as uric acid or guanine could be stored in the kidney and only released at death. Duerr (1966), in a study on Limnaea stagnalis, found that these snails excrete very little, if anything. He suggested that they store uric acid in their body and excrete this infrequently. Needham (1945) reported 115 mg uric acid/g dry kidney wt for L. stagnalis, which has a life span of 14 months (Noland and Carricker, 1946).

An attempt was made in the present study to determine the life span of Mesomphix by weighing and measuring collected snails, but the results were inconclusive. It appeared, however, that by early spring almost all of the

large snails living through the winter had died. At about the same time very small snails had begun to appear. Thus there is a good possibility that Mesomphix vulgatus is annual.

Mesomphix kidney contains a significantly higher percentage of guanine nitrogen than does Duglandina kidney ($p < 0.01$, t test). Guanine is a more efficient storage purine than uric acid, since it contains 25% more nitrogen per molecule. Guanine is also less soluble than uric acid. The solubility of uric acid is $0.0025 \text{ g}/100 \text{ ml H}_2\text{O}$ at 20°C , while that of guanine is $0.0005 \text{ g}/100 \text{ ml H}_2\text{O}$ at 20°C (Albert and Brown, 1954). By the storage of large amounts of guanine, Mesomphix is able to store more nitrogen in its kidney, with less energy used in its deposition than if it used uric acid alone.

Other aspects of Mesomphix bear on its nitrogen storage. It is a scavenger, feeding on dead snails and decaying vegetable matter. Therefore it ingests more nitrogen per unit weight of food than does a strict vegetarian. This may necessitate excretion of stored purines on rare occasions. However, Mesomphix is not a very active snail. It spends much of the time withdrawn into its shell. Only after a rain was it possible to find numerous active snails. While inactive, very little metabolism is likely to occur, keeping nitrogen catabolism to a minimum.

It would be desirable to determine whether there is an increase in the amount of TNPN stored in the kidney of Neomphix over the period of a year.

Englandina, a much larger snail and a rapacious carnivore, ingests appreciable amounts of protein nitrogen. However, large quantities of excreta are voided within 12 hours after feeding, thus eliminating excess nitrogen.

Uric acid is the major purine found in Englandina kidney and excreta. Although there is an apparent difference in the percent of uric acid in the kidney and the excreta, the difference is mostly due to the amino acid nitrogen found in the kidney. If amino acids are disregarded, the percent of uric acid nitrogen of Englandina kidney closely approximates that of excreta. Guanine is also a significant material in both kidney and excreta, but does not represent as high a percentage of the TNPN in Englandina as it does in Neomphix. Nucleic acid catabolism and ingested guanine from prey kidney can probably account for most guanine found in Englandina.

Several previous studies have shown the presence of three purines in snail kidney and excreta: uric acid, guanine, and xanthine, in H. pomatia (Jezewska, et al., 1963a,b) and in G. lacter (Lee and Campbell, 1964). Xanthine is approximately 25 times as soluble as uric acid (Dawson, et al., 1959), thus requiring more energy for water resorption when this material is excreted, or

resulting in the loss of more water. These two restrictions do not affect Euglandina and Mesomphix.

In snail uric acid biosynthesis, Bricteux-Gregoire and Florkin (1962) conclude that the pathway elucidated by Buchanan, et al. (1948) is operative. The pathway contains the reaction sequence, guanine ~~guanase~~ xanthine ~~xanthine oxidase~~ uric acid. Snail species excreting all three of these purines must have guanase and xanthine oxidase with fairly low activities. Xanthine oxidase of Euglandina and Mesomphix, however, must be very active, so that, on formation, xanthine is immediately converted into uric acid and does not appear in the kidney.

Ammonia and urea are present in all material examined, but do not represent appreciable excretory nitrogen. Ammonia found in the kidney may be merely blood ammonia, since the amount found in Euglandina excreta is significantly lower ($p < 0.005$, t test) and could be due to bacterial action on the excreta.

Speeg and Campbell (1968) have reported the elimination of appreciable quantities of ammonia nitrogen from O. lectes and Helix aspersa in the form of ammonia gas. The amounts from estivating snails compare favorably with amounts excreted by non-terrestrial molluscs (Potts, 1967). Ammonia gas from active snails is 50% less than that from estivating snails. Active individuals of the two species from the present work were analysed in a fashion similar

to the method of Speeg and Campbell (1968) as were several active H. aspersa. No ammonia gas was recovered, while 100% recoveries of known ammonia were obtained. It is possible that these active snails produced amounts too low to be detected, or the ammonia gas is not produced continually, none being produced at the time of analysis.

Euglandina excretory amino acid nitrogen is very low when compared with literature values, such as the 5.2% TNPN in H. pomatia found by Delaunay (1931). However, Jezewska, et al. (1963a) found no amino acids in either kidney or excreta of H. pomatia.

It is evident that amino acids are not an important constituent of Euglandina excreta. They are a very important fraction of kidney nitrogen, however, in both Euglandina and Mesomphix. There are few data available on amino acid content of kidneys or other land snail organs. Florkin (1960) found 212.2 mg free amino acids/100 g of H. pomatia hepatopancreas. Kerkut and Cottrell (1962) reported 1 M amino acids/ml serum and 18.2 M/g wet weight of brain of H. pomatia. Filson (1965) found 4.9 - 9.6 mg non-protein nitrogen/100 ml serum in various species of Maliotis. These data are difficult to compare with those of the present study, but reported values seem to be lower than those found here.

The pattern of amino acids present in Euglandina kidney and excreta, and that of Mesomphix is very similar.

Awapara (1962) reports Engelhardina singlyana and its herbivorous prey Eulimulus also show relatively little difference in their amino acid patterns. One significant difference was noted between Engelhardina and Mesomphix in the present work. Histidine was present in Engelhardina kidney and excreta in large amounts, as evidenced in intensity of staining on the chromatogram. Mesomphix kidney contained large amounts of phenylalanine, with much less histidine. No reason for this difference is immediately evident. The function of the high amino acid levels found in snails, as well as in other invertebrates, is unknown except in those where it is correlated with osmotic regulation.

The pattern of nitrogen excretory products of the species studied here is similar in many respects to previously studied snails. Purines make up the bulk of the nitrogen. Uric acid is preponderant, but guanine is present in significant amounts. Ammonia, urea and amino acids are found in low quantities in Engelhardina excreta. Kidneys of both species contain little ammonia and urea, but large amounts of amino acids. Both species rely heavily on the storage capabilities of their kidneys, Mesomphix doing so to the exclusion of excretion. The pattern of nitrogen compounds is remarkably similar in the two snails, one a carnivore, the other a scavenger and prey of the former. Differences are attributable to longevity and

possibly to amount of protein in the diet of the respective species.

It has become increasingly evident that land snails are "purinotelic" and not just uricotelic. The study is and interpretation of snail nitrogen excretion should be attempted only with the realization that several different purines may be involved.

SUMMARY

1. The kidneys and excreta of two pulmonate land snail species, Mesomphix vulgatus and Euglandina rosea, were analysed for non-protein nitrogen components.
2. Mesomphix was found to void no excretory material.
3. The purines, uric acid and guanine, represent 64.9% of the total non-protein nitrogen (TNPN) of Mesomphix kidney TNPN, 63.9% of Euglandina kidney TNPN, and 89.8% of Euglandina excreta TNPN.
4. Mesomphix kidney contained 42.9% uric acid and 22.0% guanine. Euglandina kidney was 55.9% uric acid and 8.0% guanine, while the excreta was 82.9% uric acid and 6.9% guanine.
5. Amino acids represented 24.7% of the TNPN of Mesomphix kidney and 19.3% of Euglandina kidney. Euglandina excreta contained only 0.6% of the TNPN as amino acids.
6. Urea and ammonia were present in the kidneys and excreta, but both materials together represented less than 4% of the TNPN. No gaseous ammonia was released by either species.
7. Almost all of the TNPN of the kidneys and excreta was accounted for: 93.3% in Mesomphix kidney, 86.6% in Euglandina kidney, and 92.0% in Euglandina excreta.

8. It was considered that Mesomphix vulgaris is a short-lived species and an individual may excrete very little if any nitrogen during its lifetime.
9. The kidney of Mesomphix contains a significantly higher percent TNPN as guanine than does Englandina kidney. This allows more nitrogen to be stored with an expenditure of less energy per molecule of purine, enabling Mesomphix to rely on storage of nitrogen rather than excretion.
10. Englandina, while able to store large amounts of nitrogen in the kidney, mainly as uric acid, voids waste nitrogen excretory products within 12 hours of feeding.
11. The term "purinotelic," rather than uricotelic, seems a better one to apply to land snail excretion.

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BIOGRAPHICAL SKETCH

David George Badman was born November 30, 1941, at Chippewa Falls, Wisconsin; he was graduated from Chippewa Falls Senior High School. He attended the University of Wisconsin, and in June, 1963, he received the degree of Bachelor of Science in Zoology. In September, 1963, he enrolled in the Graduate School of the University of Florida. He worked as a graduate assistant in the Department of Zoology until August, 1969. He received the degree of Master of Science with major in Zoology in August, 1966. From September, 1966, until the present time he has pursued his work toward the degree of Doctor of Philosophy. He spent the spring and summer of 1965 at Friday Harbor Laboratories, University of Washington, Friday Harbor, Washington, supported by a National Science Foundation Fellowship.

David George Badman is married to the former W. Sue Griffith. He is a member of the Phi Sigma Society, The Society of the Sigma Xi, the American Association for the Advancement of Science, and the American Society of Zoologists.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1969

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